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Pharmacologic Effects of Fractions of Oxidized Oleate and Linoleate

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Purified ethyl oleate and methyl linoleate were autoxidized at 95 C. in an oxygen current for 240 and 100 hours, respectively. After saponification and re-esterification with ethyl alcohol, the ethyl esters were fractionated by alembic and molecular distillations in conjunction with urea segregation. Two fractions of the molecular distillate and three from the residue of the autoxidized oleate, and four fractions from the molecular distillation residue of the linoleate, were obtained in sufficient quantities for feeding tests. Chemical characterization was based on determinations of acid, saponification, iodine, hydroxyl and carbonyl numbers.

The fractions were fed at a level of 8 per cent (4 per cent in some cases) in a purified diet to weanling male rats for three weeks. Survival rate, growth, water intake, organ weights, total liver lipids, and liver and serum cholesterol levels were recorded.

Some of the fractions exerted characteristic effects on growth, diuresis, organ weights and cholesterol metabolism. The urea-adduct–forming fraction of the oleate distillate, which consisted of mono- and di-basic, saturated, short chain esters, was atoxic and yet, in comparison to lard, produced marked diuresis and reduced liver and serum cholesterol levels.

Considerable interest has been given to the changes occurring in fats and oils during heating and aeration.[1-3] It seemed a foregone conclusion that any changes in the fats must necessarily be undesirable; therefore, most interest has been centered in attempts to avoid such changes. On the other hand, during heating and oxidation of fats, many structurally unknown substances are developed, and one may ask whether any of these substances has effects which, under certain conditions, would be beneficial.

In previous work, we studied fractions of oxidized lard and cottonseed oil obtained by a urea-complex separation and distillation. In the current studies, we selected for study fractions of oxidized methyl linoleate and ethyl oleate to obtain better-characterized fractions.

Materials and Methods

The preparation of the oxidized ethyl oleate fractions is summarized in figure 1. Ethyl oleate with an iodine number of 80.5, and containing less than 1 per cent polyunsaturated esters and about 2 per cent saturated esters, was prepared from olive oil fatty acids by low-temperature crystallizations and fractional vacuum distillation. This material was oxidized with well dispersed streams of oxygen at 95 to 100 C. for 240 hours. The iodine

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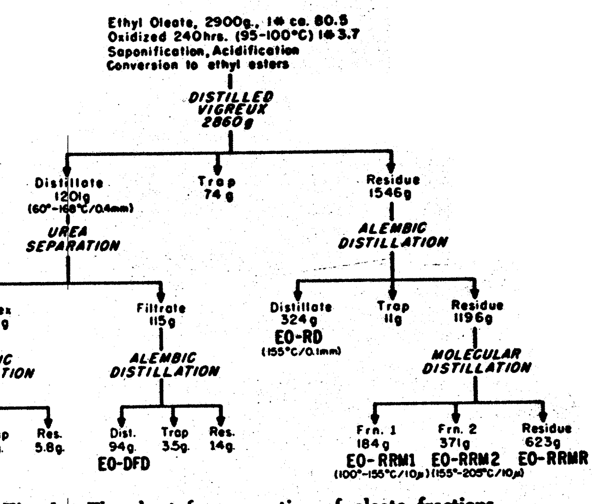

Fig. 1.—Flowsheet for separation of oleate fractions.

number of the oxidation product was 3.7. The oxidation mixture was saponified with aqueous NaOH, acidified to the free acids and esterified with absolute ethyl alcohol. Fractional vacuum distillation of these esters through a 2" x 20" Vigreux column gave 42 per cent distillate (boiling range, 60 to 168 C./0. 4 mm.) and 54 per cent residue.

The distillate was separated into four fractions by urea segregation in methanol solution and subsequent high-vacuum, short-path distillations of the complex and filtrate through an alembic type apparatus. Only the distillates (32 per cent of the urea-complex–forming, and 4 per cent of the noncomplex-forming fractions) were sufficient in quantity for feeding tests.

A short-path, high-vacuum distillation of the residue from the Vigreux distillation gave a distillate, EO-RD (11 per cent, collected up to 155 C./0.1 mm.), and a residue, which was molecularly distilled to give the following fractions: EO-RRM1 (6 per cent, collected at 100 to 155 C./10 μ), EO-RRM2 (13 per cent, 155–205 C./10 μ) and a residue, EO-RRMR (22 per cent).

The preparation of the oxidized methyl linoleate fractions is summarized in figure 2. Methyl linoleate, with an iodine number of 170.2 and about 97 per cent pure, was prepared by methanolysis of safflower seed oil, selective crystallization from a methanol solution of urea to remove the oleate and saturated esters and fractional distillation. The methyl linoleate oxidation was carried out at 90 to 95 C. in the same way as the ethyl oleate but was terminated after 100 hours. The iodine number of the oxidized product was 51.5. This material was saponified, acidified and esterified with absolute ethanol. Fractional distillation of these ethyl esters yielded a distillate too small for further separation and feeding studies.

Molecular distillation of the residue gave a fraction, collected at 100 to 150 C./12 μ, which was separated into a urea-complex–forming fraction, EL-RM1C (7.3 per cent), and a noncomplex-forming filtrate, EL-RM1F (6 per cent). The nonvolatile residue, EL-RMR, amounted to 55 per cent.

The results of chemical analyses of these fractions are summarized in table 1. Gas-phase chromatography of the volatile fractions showed that they contained numerous compounds. From the high saponification numbers of the oleate fractions, it is apparent that considerable cleavage of the carbon chains had occurred. EO-DCD and EO-DFD must have contained

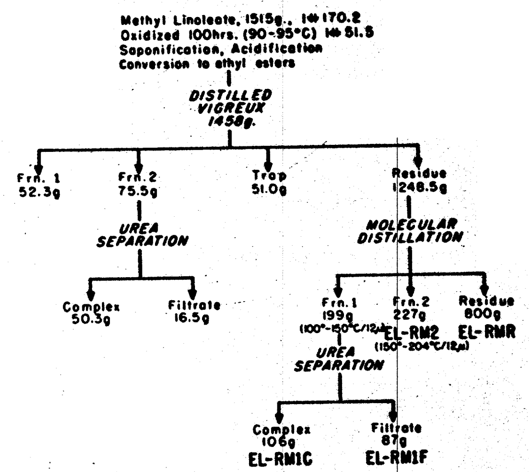

Fig. 2.—Flowsheet for separation of linoleate fractions.

Table 1.—*Analysis of the Ethyl Esters Obtained by Fractionation of Oxidized Ethyl Oleate and Linoleate*

Sample	Acid No.	Sapon. No.	Iodine No.	% Hydroxyl Oxygen	% Carbonyl Oxygen	Comments
EO-DCD	4.6	370	2.3	0.3	0.5	mono- and di-basic esters
EO-DFD	109.5	328	7.0	2.8	0.8	oxygenated mono- and di-basic acids and esters
EO-RD	59.4	281	65.0	0.4	1.2	acid chain length of C-18 or less
EO-RRM1	27.2	278	51.3	0.5	1.5	mainly monomeric esters, some dimer
EO-RRM2	27.8	288	34.0	0.9	1.1	mostly dimer esters
EO-RRMR	22.7	287	31.6	0.5	1.1	polymers higher than dimers
EL-RM1C	19.6	234	105.0	1.1	1.9	oxygenated long chain esters, urea adduct-formers
EL-RM1F	39.1	228	76.2	1.8	1.3	oxygenated long-chain esters, non-adduct-formers
EL-RM2	29.9	225	78.1	1.2	1.1	mostly dimer esters
EL-RMR	24.3	219	78.3	1.0	0.9	polymers higher than dimers

considerable amounts of the esters of shorter chain mono- and di-basic acids. Moreover, over half of the esters of EO-DFD were hydroxylated. The major part of the carbonyl compounds were concentrated in EO-RRM1, EO-RRM2 (shown by distillation data to be the dimeric fraction), and EO-RRMR (the higher polymers). The chemical data for the linoleate fractions suggest that they were more highly oxidized and that there was little cleavage of the carbon chains. Distillation data indicated that EL-RM2 contained mostly dimers, and EL-RMR, mostly higher polymers.

The studies were carried out on well matched groups of eight weanling male rats weighing about 100 grams, which had been derived from a homogeneous colony of the Sherman strain. The fractions were incorporated at a level of 8 per cent (4 per cent where noted) in a purified diet containing 30 per cent alcohol-washed casein, 56 per cent dextrose, 4 per cent salts (USP XII), 2 per cent cellulose (Alphacel) and liberal vitamin sup-

Table 2.—*Summary of Results of Feeding Oleate Fractions*

	Chemical Characteristics	Survival Rate	Body Wt. (Gm.)	Water Intake	% Weight Deviation				
					Liver	Kidneys	Ad-renals	Thymus	Testis Fat Body
Lard		16/16	208	213	−1	0	+3	+2	9
			±2.3	±4.9	±1.8	±.9	±3.1	±3.8	±3.8
Ethyl		8/8	189	235	−15	−6	+18	−39	+10
oleate			±2.2	±22.6	±2.1	±3.4	±8.4	±6.7	±3.9
DCD	Mono- & di-basic	16/16	168	344	−5	+14	+2	−35	+16
	short, saturated		±3.1	±27.5	±3.2	±1.3	±3.3	±3.6	±7.6
	add.-form. esters								
DFD	Oxygenated mono-	4/4	131	265	−2	+34	+38	−38	+25
	& di-basic acids		±9.1	±26.0	±5.5	±1.7	±4.8	±4.8	±13.1
	and esters								
RD	Acids of C_{18} and	15/16	118	316	−7	+14	+16	−57	−12
	less; C=C & C=O		±3.4	±20.2	±1.9	±2.0	±4.8	±4.0	±3.9
RRM-1	Mainly monomeric	9/10	92	211	+14	+43	+29	−73	−14
	esters		±2.7	±10.0	±4.3	±9.1	±7.3	±3.6	±7.4
RRM-2	Mostly dimeric	12/16	89	252	+28	+11	+20	−74	−21
	esters		±3.1	±12.0	±2.4	±2.2	±5.1	±3.1	±9.5
RRMR	Higher polymers	15/16	140	328	−3	+1	+11	−43	−11
			±3.7	±16.6	±1.7	±1.4	±3.7	±3.5	±8.6

Water intake is expressed as ml./100 Gm. body weight for three weeks. ± values are standard errors.

plements.* Control groups received 8 per cent lard, ethyl oleate and ethyl linoleate. Growth, survival rate, organ weights and water consumption were measured; details of the procedure have been published elsewhere.[4]

After three weeks, the animals were anesthetized; heart blood was obtained for cholesterol analyses, which were carried out on conveniently pooled samples according to the method of Schoenheimer and Sperry.[5] Organs were weighed; the livers were frozen for subsequent dry weight, total lipid and cholesterol determinations, which were carried out on pooled samples of 2 to 4 livers. Liver extractions were done according to the method of Neift and Deuel,[6] and cholesterol determinations according to that of Sperry and Webb.[7] Two complete series of experiments were carried out, the results of which proved to be very similar and were therefore combined in the tables.

Organ weights were evaluated in relation to the body weight. With normal animals, a log-log plot of this relationship is a straight band, the slope and width of which are characteristic for each organ; the slope may show one or more changes with increasing body weight.[8] We obtained these plots by using a long series of normal animals of widely varying body weights.[3] The weight of each organ of the animals which served as controls in the current experiments was plotted on log-log paper, and the best straight line with the established slope for the organ was drawn through them. The observed organ weights were compared with the "ideal" weights read from this line at the same body weight, and the differences were expressed as percentages of the ideal weight. Thus, even the control groups could show slight deviations from the ideal value.

For statistical analysis, standard errors are given, from which t and p values can be calculated. A p of .05 was considered to be just significant.

Results

From the results with the oleate fractions given in table 2, it is evident that the most severe weight depressions were induced by the monomers

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and dimers (RRM-1 and RRM-2) but that most of the animals survived the three week period. The higher polymers (RRM-R) permitted better growth. The fraction DCD, containing urea adduct-forming, mono- and dibasic short-strain, naturated esters promoted growth almost as well as did ethyl oleate.

Interpretation of the water intakes was difficult because of the variations in body weight. To obtain some degree of comparability, the total water intake of each animal for the three week period was divided by the average of its weight at the beginning and end of the test period, and the result was multiplied by 100. Thus, the data on water intake in the table are given as ml. per 100 grams body weight. Fractions DCD, RD and the higher polymers induced high intakes.

Studies of the organ-body weight relationship showed that the monomeric and dimeric esters caused significant enlargement of the livers, kidneys and adrenals and atrophy of the thymus. The monomers induced particularly large kidneys and relatively smaller livers; the dimers did the opposite (the differences were significant).

The weight of the fat body next to the testes was studied because it has been shown to be proportional to the amount of neutral fat present in the rat.[9,10] For normal animals, we have found that the slope of the distribution for body weights above 100 grams is 64 degrees; the weight of the fat body at 100 grams of body weight was 0.39 ± 0.10 grams. The fat bodies of the groups on fractions of the original distillate (DCD, DFD) seemed relatively heavier than those of the lard group; but only if the two groups were combined was the difference on the borderline of significance. All fractions of the original residue gave relatively small fat bodies. However, only RD and the dimeric fraction (RRM-2) gave statistically significant results in comparison to lard; all differences were statistically significant with respect to DCD and DFD.

Results with the ethyl linoleate fractions are summarized in table 3.

Table 3.—*Summary of Results of Feeding Linoleate Fractions*

	Chemical Characteristics	Survival Rate	Body Wt. (Gm.)	Water Intake	% Weight Deviation: Liver	Kidneys	Adrenals	Thymus	Testis Fat Body
Lard		16/16	208 ±2.3	213 ±4.9	−1 ±1.8	0 ±.9	+3 ±3.1	+2 ±3.8	0 ±2.8
Ethyl linoleate		8/8	168 ±7.4	218 ±12.3	−6 ±2.6	+4 ±3.1	+22 ±6.0	−28 ±7.1	−19 ±4.7
RM1C	Oxygenated, long chain, add.-form. esters	5/5	91 ±5.7	379 ±55.7	+12 ±4.2	0 ±4.9	+36 ±2.2	−66 ±6.1	+14 ±14.4
RM1F*	Ox. long-chain non-add. form. esters	4/4*	91 ±1.4	263 ±57.6	+14 ±5.3	+21 ±6.8	+35 ±10.0	−75 ±3.2	−30 ±4.1
RM-2*	Mostly dimers	6/8*	102 ±2.5	283 ±33.5	+16 ±.9	+4 ±1.8	+31 ±6.9	−71 ±3.5	+16 ±5.5
RMR	Higher polymers	15/16	119 ±4.8	253 ±14.0	−6 ±2.3	+1 ±2.3	+14 ±4.5	−48 ±3.3	+21 ±5.8

Water intake is expressed as ml./100 Gm. body weight for three weeks. ± values are standard errors.
*Fed at a level of 4%.

The oxygenated, long-chain, nonadduct-forming esters (RM1-F) and the dimers (RM-2) were so toxic that all of the animals receiving 8 per cent died rapidly, in contrast to the animals fed comparable fractions from oleate. With 4 per cent, most animals survived, but weight was depressed severely. The higher polymers (RM-R) and the oxygenated, urea-adduct–forming, long-chain esters (RM1-C) permitted survival at the 8 per cent level but depressed weight severely. The latter was especially potent in depressing weight and also induced high water intakes.

The dimers significantly increased the relative weight of the liver but not of the kidneys. Kidney weight was particularly affected by RM1-F. The testicular fat body weights suggested that the oxygenated, long-chain, nonadduct-forming esters reduced neutral fat deposition, and the higher polymers increased it. The latter finding is opposite to that with the oleate higher polymers, which decreased neutral fat deposition.

In table 4 are summarized the lipid studies with oleate fractions. Total liver lipid and cholesterol contents are given on the basis of dry weight. Determinations of the dry residue indicated no significant differences for the various fractions. The enlarged livers of the animals on the monomeric and dimeric esters had a low total lipid content; the enlargement was therefore the result of protein accumulation. The fractions produced low liver and serum cholesterol levels. Ethyl oleate gave the highest liver values and a relatively high serum level. Interesting was the result with the relatively atoxic mono- and di-basic, short-chain, saturated, urea-adduct–forming esters (DCD), which produced a low liver cholesterol level but the highest serum level of any of the fractions of oxidized oleate. This is in line with observations in feeding studies with the triglycerides of medium-length chain, naturated fatty acids previously reported.[11]

Table 4.—*Summary of Lipid Studies with Oleate Fractions*

	Chemical Characteristics	Total Liver Lipids (% dry wt.)	Liver Cholesterol (mg.% dry wt.)	Serum Cholesterol (mg.%)
Lard		24.3 ±.9	861 ±42	86 ±5.0
Ethyl oleate		25.9 ±1.0	1053 ±19	71 ±8.0
DCD	Mono- & di-basic, short-chain, saturated, add.-form. esters	24.1 ±.9	765 ±24	59 ±5.6
DFD	Oxygenated mono- & di-basic acids and esters	23.0 ±.2	857 ±47	47 ±4.5
RD	Acids of C_{18} or less; C=C and C=O	24.3 ±1.1	841 ±21	54 ±5.9
RRM-1	Mostly monomeric esters	21.7 ±.3	780 ±73	45 ±4.5
RRM-2	Mostly dimeric esters	22.2 ±.7	753 ±13	44 ±2.7
RRMR	Higher polymers	22.9 ±1.1	839 ±25	54 ±4.4

± values are standard errors.

Table 5.—*Summary of Lipid Studies with Linoleate Fractions*

	Chemical Characteristics	Total Liver Lipids (% dry wt.)	Liver Cholesterol (mg.% dry wt.)	Serum Cholesterol (mg.%)
Lard		24.3 ±.9	861 ±42	86 ±5.0
RM1C	Oxygenated, long chain, add.-form. esters	21.5 ±4.0	873 ±8	49 ±5.0
RM1F*	Oxygenated, long chain, non-add.-form. esters	26.1	835	43
RM-2*	Mostly dimers	23.9 ±1.0	791 ±4	65 ±0
RMR	Higher polymers	22.7 ±.9	844 ±14	50 ±6.0

± values are standard errors.
*Fed at a level of 4%.

In table 5 are summarized the lipid values obtained after the feeding of oxidized linoleate fractions. As with the oleate fractions, serum cholesterol levels were much lower than with lard. Again, the toxic dimer fraction produced low liver cholesterol levels. The higher polymers of oxidized oleate and linoleate gave very similar results as to total liver lipid and serum and liver cholesterol.

Discussion

Autoxidation of ethyl oleate and methyl linoleate led to the formation of a large number of hitherto untested organic compounds. Some of these substances must be of pharmacologic interest because fractions from the autoxidized materials, although they contained many chemical entities, gave distinct effects when fed to rats.

Water intake measurements showed that oleate fractions, DCD, RD and the higher polymers, and the linoleate fraction, RM1-C, induced higher intakes and must therefore have had a diuretic effect.

Some of the fraction led to enlargement of individual organs. This organ specificity suggests that the effects were not due to nonspecific stress.

Definite effects on lipid metabolism were observed with some of the fractions. Some of them depressed neutral fat deposition. The more toxic fractions led to low liver and serum cholesterol levels, which suggests decreased cholesterol synthesis. One fraction of the oxidized oleate, DCD, which consisted of saturated, short-chain, mono- and di-basic esters, was essentially atoxic but was associated with low liver cholesterol values.

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